# Chemically Modified Chitosans as Antimicrobial Agents against Some Plant Pathogenic Bacteria and Fungi

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**Abstract**

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The antimicrobial activity of eight chemically modified chitosans against plant pathogenic bacteria of crown gall disease *Agrobacterium tumefaciens* and soft mould disease *Erwinia carotovora* and fungi of early blight disease *Alternaria alternata*, root rot disease *Fusarium oxysporum*, and damping off disease *Pythium debaryanum* was investigated. The minimum inhibitory concentration (MIC) of these compounds against the tested bacteria showed that *N*-(*o*,*o*-dichlorobenzyl)chitosan exhibited a high activity against *A. tumefaciens* and *E. carotovora* (MIC = 500 and 480 mg/l, respectively). In addition, the antifungal behaviour was investigated *in vitro* on mycelial growth and spore germination. *N*-(*p*-fluorobenzyl)chitosan was the most active against the mycelial growth of *A. alternata* with an $EC_{50}$ of 703.4 mg/l, while the *N*-(*o*-chloro,*o*-fluorobenzyl)chitosan exhibited a high activity against *F. oxysporum* and *P. debaryanum* ($EC_{50}$ = 641.2 and 155.7 mg/l, respectively). Against the fungal spore germination, all modified chitosans showed a higher inhibition of spore germination than unmodified chitosan. The compound of *N*-(*o*,*o*-di fluorobenzyl)chitosan exhibited a high inhibition percentage against *A. alternata*. However, *N*-(*o*-chlorobenzyl)chitosan and *N*-(*p*-chlorobenzyl)chitosan were significantly highly active against the spore germination of *F. oxysporum*.

**Keywords**: chemically modified chitosans; antibacterial activity; antifungal activity; spore germination

Plant pathogenic bacteria and fungi are considered economically important around the world. They induce decay on a large number of economically important agricultural crops during the growing season and during postharvest storage. Control of this disease is especially important and can be achieved by synthetic pesticides. However, there are growing environmental problems caused by bactericides and fungicides, especially by synthetic products (Carson 1962; Houeto *et al.* 1995).

In recent years there has been an increasing interest in finding alternatives to chemical bactericides and fungicides considered as safe, and with negligible risk to human health and environment. Among these strategies, some satisfactory results have been reported using natural compounds such as chitosan (Muzzarelli 1983). A biopolymer of chitosan is one of the most abundant naturally occurring amino-polysaccharides of glucosamine and *N*-acetylglucosamine and it has attracted attention because of its unique physiochemical characteristics and biological activities (No & Meyers 1997). Amongst various bioactive properties of chitosan, its antimicrobial activity has received considerable interest due to problems associated with fungicidal agents (Roller & Covill 1999; Guo *et al.* 2006). Furthermore, numerous studies on the antimicrobial activity of chitosan and its derivatives against plant pathogens have been carried out (Allan & Hadwiger 1979; El-Ghaouth *et al.* 1992, 1994;

Baba *et al.* 1996; Reddy *et al.* 2000; Badawy 2008; Rabea *et al.* 2009) and reviewed (Rabea *et al.* 2003; Bautista-Baños *et al.* 2006). Its inhibitory effect was observed on different development stages such as mycelial growth, sporulation, spore viability and germination, and the production of fungal virulence factors (Xu *et al.* 2007).

The natural antibacterial and/or antifungal characteristics of chitosan and its derivatives (Papineau *et al.* 1991; Sudarshan *et al.* 1991; El-Ghaouth *et al.* 1992; Kim *et al.* 1997; Chung *et al.* 2004) have resulted in their use in commercial disinfectants. Chitosan has several advantages over other types of disinfectants because it possesses a higher antibacterial activity, a broader spectrum of activity and a lower toxicity for mammalian cells (Liu *et al.* 2001).

The scope of this study was to compare the antimicrobial activities of chitosan with differently modified chitosans against plant pathogenic bacteria of crown gall disease *Agrobacterium tumefaciens* and soft mould disease *Erwinia carotovora* and fungi of early blight disease *Alternaria alternata*, root rot disease *Fusarium oxysporum* and damping off disease *Pythium debaryanum*.

## MATERIALS AND METHODS

***Chemicals and media for bioassay***. Low-molecular-weight chitosan (made from coarse ground crab, 89% degree of deacetylation) was supplied by Sigma-Aldrich Co. (St. Louis, USA). Eight chemically modified chitosan derivatives, listed below in Figure 1, were prepared and characterized as described by Rabea *et al.* (2005). The compounds with the indicated functional groups and different substituents were the following: compound 1, *N*-(*p*-chlorobenzyl)chitosan, compound 2, *N*-(*o*-chlorobenzyl)chitosan, compound 3, *N*-(*p*-fluorobenzyl)-chitosan, compound 4, *N*-(*o*-fluorobenzyl)chitosan, compound 5, *N*-(*o*,*p*-dichlorobenzyl)chitosan, compound 6, *N*-(*o*,*o*-dichlorobenzyl)chitosan, compound 7, *N*-(*o*,*o*-difluorobenzyl)chitosan and compound 8, *N*-(*o*-chloro,*o*-fluorobenzyl)-chitosan. Potato Dextrose Agar (PDA), Nutrient Broth (NB) and Nutrient Agar (NA) media were purchased from Sigma-Aldrich Co. (St. Louis, USA) and all materials were used without further purification.

***Test microorganisms***. Bacteria of crown gall disease *Agrobacterium tumefaciens* (Family: Rhizobiaceae; Class: Alpha Proteobacteria) and soft mould disease of *Erwinia carotovora* (Family: Enterobacteriaceae; Class: Gamma Proteobacteria) and fungi of early blight disease *Alternaria alternata* (Family: Dematiaceae), root rot disease *Fusarium oxysporum* (Family: Tuberculariaceae; Class: Deuteromycetes) and damping off disease *Pythium debaryanum* (Family: Pythiaceae; Class: Oomycetes) were provided by Microbiology Laboratory, Department of Plant Pathology, Faculty of Agriculture, Alexandria University, Alexandria, Egypt.

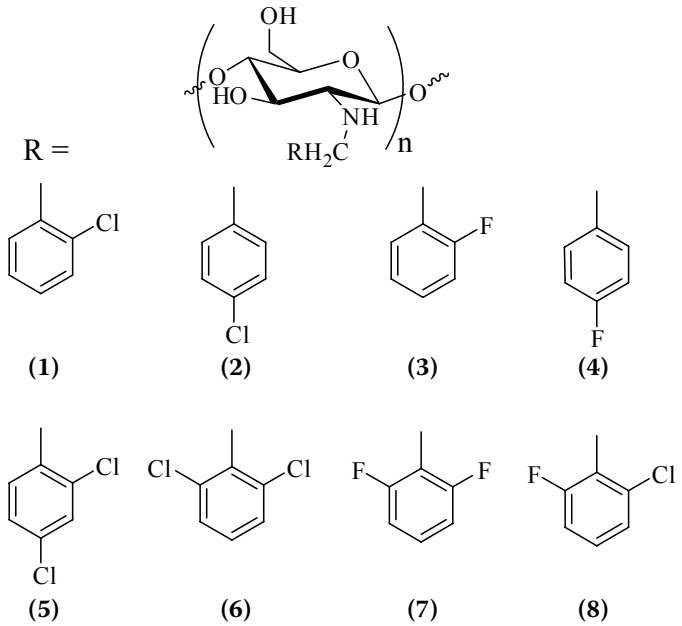



Figure 1. Chemical structure of modified chitosans

***Antibacterial bioassay***. The antibacterial activity of chitosan derivatives was assayed using the nutrient agar dilution method as recommended by EUCAST (2000) against *A. tumefaciens* and *E. carotovora*. For MIC determination, Nutrient agar (NA) medium was mixed with a solution (0.5%, v/v, lactic acid) of the compounds aiming at the final concentrations ranging from 200 mg/l to 2400 mg/l, and were then poured into autoclaved Petri dishes. One loopful of microorganism suspensions in Nutrient Broth (NB) medium (6 µl) was spotted (10 spots/plate) and then incubated at 37°C. Each concentration was tested in triplicate. The MIC was determined as the lowest concentration of the chitosan derivatives required to completely inhibit bacterial growth (100 %) after incubation at 37°C for 24 hours.

***Antifungal bioassay***. Antifungal assay was performed on agar plates amended with chitosan compounds for the radial mycelial growth determination of plant pathogenic fungi *A. alternate, F. oxysporum*, and *P. debaryanum.* Different concentrations of chitosans (100, 250, 500, 1000, 2000, and 3000 mg/l) were added to sterilised PDA medium. The discs of mycelial culture of the tested fungi were transferred aseptically to the centre of Petri dishes. The test plates were incubated in the dark at 28°C. When the mycelium reached to the edges of the control plate, the radial colony growth measurements were determined and the effective concentration causing a 50% inhibition of mycelial growth ($EC_{50}$) with corresponding 95% confidence limits was estimated by a probit analysis (Finney 1971).

### Effect of chitosans on spore germination of *A. alternata* and *F. oxysporum* in liquid medium

***Preparation of fungal spores.*** Spore suspensions of *A. alternata* and *F. oxysporum* were obtained from their respective 2-weeks-old PDA culture grown under fluorescent lights in Petri dishes at 26°C. In this study, the spore germination assay was not evaluated on *P. debaryanum* because this fungus forms various types and shapes of oospores and sporangia (bearing conidia). An aliquot of 5 ml of sterile distilled water was added to a Petri plate culture. The spores were gently dislodged from the surface with a sterile glass rod and the suspension was filtered through three layers of cheesecloth to remove mycelia fragments. The suspension was diluted with sterile distilled water to an absorbance of 0.25 nm at 425 nm as determined by a spectrophotometer. This suspension contained about $1.0 \times 10^6$ spores/ml.

***Spore germination assay***. Fifty microlitres of spore suspension were transferred to an Eppendorf tubes containing 500 µl of liquid medium with different concentrations of treatments (125, 250, and 500 mg/l). The samples were incubated at 25 ± 2°C for 16 hours. Spores were considered to be germinated when the germ tube extended to at least twice the length of the spore itself (Griffin 1994). Germinated spores were counted using a Neubauer haemocytometer and light microscopy at 40×. The samples were placed on both chambers of the haemocytometer by carefully touching the edges of cover slip with the pipette tip and capillary action was allowed to fill the counting chambers that were observed under the microscope for spore germination. All experiments were conducted in four replications.

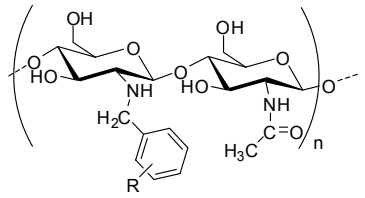
***Statistical analysis***. Statistical analysis was performed using a statistical software program (SPSS Version 12.0, Chicago, USA) according to probit analysis (Finney 1971). The log dose-response curves allowed to determine $EC_{50}$ in fungi bioassay. The 95% confidence limits (CL) and standard error for the range of $EC_{50}$ values for the compound for assays were determined by the least-square regression analysis of relative growth rate (% control) against the logarithm of the compound concentration. The data on spore germination was analyzed by one-way analysis of variance (ANOVA). Mean separations were performed by the Student-Newman-Keuls (SNK) test and differences at $P < 0.05$ were considered as significant.

## RESULTS AND DISCUSSION

### Antibacterial activity of chitosan compounds

The antibacterial activity of chitosan compounds is shown in Table 1 as MIC. Chitosan derivatives markedly inhibited the growth of the two bacteria with MIC values ranging between 480 mg/l and 1700 mg/l and their activity were higher than that of unmodified chitosan. The inhibitory effects differed with regard to the substituents and the type of bacteria. *N*-(*o*,*o*-dichlorobenzyl)chitosan displayed the highest antibacterial activity against *A. tumefaciens* and *E. carotovora* with MIC 500 mg/l and 480 mg/l, respectively. However, *N*-(*p*-chlorobenzyl)chitosan possessed a weak

Table 1. Minimum inhibitory concentration (MIC, mg/l) of chitosan and its derivatives against the bacteria *A. tumefaciens* and *E. carotovora*



| Compound | R | DS | MIC (mg/l) of bacteria | |
|---|---|---|---|---|
| | | | *A. tumefaciens* | *E. carotovora* |
| Chitosan | – | – | > 2400 | 1350 |
| 1 | *o*-Cl | 0.41 | 850 | 600 |
| 2 | *p*-Cl | 0.39 | 1700 | 1125 |
| 3 | *o*-F | 0.34 | 725 | 550 |
| 4 | *p*-F | 0.14 | 675 | 600 |
| 5 | *o,p*-di Cl | 0.52 | 800 | 525 |
| 6 | *o,o*-di Cl | 0.33 | 500 | 480 |
| 7 | *o,o*-di F | 0.28 | 775 | 500 |
| 8 | *o*-Cl, *o*-F | 0.51 | 1000 | 900 |

DS – degree of substitution was calculated from the ratio of the areas of the protons in benzyl moiety and the protons of the pyranose unit (Rabea *et al.* 2005)

antibacterial activity against *A. tumefaciens* and *E. carotovora* (MIC = 1700 mg/l and 1125 mg/l, respectively). Considering the susceptibility of the microorganisms, it was noticed that *E. carotovora* was more susceptible to these compounds than *A. tumefaciens*. In addition, the antibacterial activity of modified chitosans depended upon the position of fluorine and chlorine atoms on the phenyl ring. An introduction of fluorine atom into the *ortho-* or *para*-position greatly increased their activities compared to the substitution of chlorine except compound 6. After the substitution of fluorine and chlorine to the phenyl ring the bactericidal activity was lower than that obtained with dichloro or difluoro substituents (see compounds 5, 6, and 7 versus 8).

The result obtained in this study coincides with that recently published (Rabea *et al.* 2009; Badawy 2010), where *E. carotovora* was more susceptible to these compounds than *A. tumefaciens*. Our previous work also showed that MIC of different *N*-(benzyl)chitosan derivatives ranged from 500 mg/l to 1100 mg/l against *E. carotovora* and from 1050 mg/l to 1500 mg/l against *A. tumefaciens*. In detail, a higher activity was obtained with *N*-(*o*-ethylbenzyl)chitosan with a MIC of 500 mg/l against *E. carotovora*, and *N*-(*o,p*-diethoxybenzyl) chitosan against *A. tumefaciens* with a MIC of 1050 mg/l (Rabea *et al.* 2009).

The molecular mechanisms of the antimicrobial activities of chitosan and its derivatives have not been determined completely yet. The effect of chitosan on microorganisms is considered to be complex. At the initial stage, chitosan, like other bactericidal polycations, binds with negatively charged components on the bacterial surface, via electrostatic interactions (Helander *et al.* 2001; Raafat *et al.* 2008). It changes the permeability of the bacterial wall and allows chitosan to access to internal cell targets, shutting down the cell division and leading to fatality (Je & Kim 2006).

***Antifungal activity of chitosan compounds***. The antifungal activity of chitosan compounds towards the three plant pathogenic fungi *A. alternata*, *F. oxysporum*, and *P. debaryanum* was investigated *in vitro* and the results are shown in Table 2. In general, all the modified derivatives were more active than unmodified chitosan and exhibited a good fungicidal activity against the tested fungi.

Against the fungus *A. alternata* (Table 2), the results indicated that *N*-(*p*-fluorobenzyl)chitosan and *N*-(*o,o*-dichlorobenzyl)chitosan were significantly the highest in activity with $EC_{50}$ of 703.4 mg/l and 773.5 mg/l, respectively, while *N*-(*o*-chlorobenzyl)-chitosan was the lowest one ($EC_{50}$ of 1315 mg/l). Chitosan without modification showed the lowest activity with $EC_{50}$ of 2338 mg/l. It can be seen from the data that the *para*-position with substitution of chlorine and/or fluorine atoms exhibited a higher activity than the *ortho*-position with the same substitution (compounds 2, 4, and 5 versus 1, 3, 7, and 8). Furthermore, the di-*ortho*-fluorine substituent was still less active than the *para*-fluorine substituent (see compound 4 versus 7).

The data in Table 2 indicated that a high inhibitory effect against *F. oxysporum* was observed in *N*-(*o*-chloro,*o*-fluorobenzyl)chitosan ($EC_{50}$ = 641.2 mg/l) but the lowest one was in *N*-(*p*-fluorobenzyl)chitosan ($EC_{50}$ = 1138 mg/l) except for the unmodified chitosan ($EC_{50}$ = 2910 mg/l). In regard to the effect of substituent and position on the phenyl moiety, it can be seen that the chlorine atom markedly enhanced the antifungal activity compared to the substitution of the fluorine atom. In addition, the di-chlorine substitution enhanced the activity more markedly than the

Table 2. Inhibition of mycelial radial growth by chitosan and its derivatives against *A. alternata, F. oxysporum,* and *P. debrianum*

| Compound | R[a] | $EC_{50}$[b] (mg/l) | 95% confidence limits (mg/l) | | Slope[c] ± SE | Intercept[d] ± S.E | $\chi^2$ |
|---|---|---|---|---|---|---|---|
| | | | lower | upper | | | |
| *A. alternata* | | | | | | | |
| Chitosan | – | 2338 | 1686 | 3519 | 2.63 ± 0.24 | −8.84 ± 0.80 | 9.02 |
| 1 | *o*-Cl | 1315 | 1110 | 1630 | 2.01 ± 0.22 | −6.26 ± 0.66 | 1.66 |
| 2 | *p*-Cl | 943.3 | 820.2 | 1079 | 2.65 ± 0.33 | −7.88 ± 0.99 | 1.07 |
| 3 | *o*-F | 1234 | 1051 | 1503 | 2.08 ± 0.22 | −6.43 ± 0.66 | 0.69 |
| 4 | *p*-F | 703.4 | 567.2 | 828.2 | 2.18 ± 0.32 | −6.22 ± 0.97 | 0.66 |
| 5 | *o,p*-di Cl | 857.1 | 729.2 | 1021 | 1.88 ± 0.21 | −5.53 ± 0.60 | 0.74 |
| 6 | *o,o*-di Cl | 773.5 | 654.3 | 921.6 | 1.82 ± 0.20 | −5.25 ± 0.60 | 1.01 |
| 7 | *o,o*-di F | 1117 | 946.8 | 1364 | 1.92 ± 0.21 | −5.86 ± 0.63 | 0.63 |
| 8 | *o*-Cl, *o*-F | 1124 | 944.6 | 1390 | 1.81 ± 0.21 | −5.54 ± 0.62 | 1.07 |
| *F. oxysporum* | | | | | | | |
| Chitosan | – | 2910 | 2301 | 4068 | 1.20 ± 0.18 | −4.14 ± 0.60 | 1.91 |
| 1 | *o*-Cl | 746.1 | 547.7 | 1035.7 | 0.98 ± 0.19 | −2.82 ± 0.55 | 2.50 |
| 2 | *p*-Cl | 809.2 | 647.8 | 969.4 | 1.88 ± 0.31 | −5.49 ± 0.94 | 1.46 |
| 3 | *o*-F | 1045 | 878.4 | 1287 | 1.76 ± 0.21 | −5.34 ± 0.61 | 1.16 |
| 4 | *p*-F | 1138 | 898.8 | 1569 | 1.28 ± 0.19 | −3.93 ± 0.57 | 2.07 |
| 5 | *o,p*-di Cl | 818.6 | 613.7 | 1137 | 1.02 ± 0.19 | −2.98 ± 0.55 | 1.82 |
| 6 | *o,o*-di Cl | 801.9 | 680 | 955.5 | 1.84 ± 0.20 | −5.36 ± 0.60 | 3.26 |
| 7 | *o,o*-di F | 958.9 | 815.6 | 1152 | 1.90 ± 0.21 | −5.66 ± 0.62 | 0.12 |
| 8 | *o*-Cl, *o*-F | 641.2 | 403.6 | 961.3 | 0.76 ± 0.18 | −2.14 ± 0.54 | 0.15 |
| *P. debrianum* | | | | | | | |
| Chitosan | – | > 3000 | – | – | – | – | – |
| 1 | *o*-Cl | 835.1 | 479.3 | 1560 | 3.06 ± 0.25 | −8.94 ± 0.75 | 6.63 |
| 2 | *p*-Cl | 1174 | 650 | 4772 | 3.0 ± 0.27 | −9.21 ± 0.81 | 8.24 |
| 3 | *o*-F | 253.7 | 94.1 | 393.2 | 0.81 ± 0.19 | −1.95 ± 0.55 | 2.55 |
| 4 | *p*-F | 196.4 | 61.1 | 320.0 | 0.83 ± 0.19 | −1.91 ± 0.56 | 0.34 |
| 5 | *o,p*-di Cl | 232.0 | 152.4 | 303.0 | 1.61 ± 0.23 | −3.81 ± 0.64 | 1.06 |
| 6 | *o,o*-di Cl | 430.1 | 181.8 | 716.7 | 3.69 ± 0.33 | −9.73 ± 0.89 | 7.51 |
| 7 | *o,o*-di F | 1142 | 761.7 | 3426 | 2.59 ± 0.24 | −7.93 ± 0.73 | 3.95 |
| 8 | *o*-Cl, *o*-F | 155.7 | 31.8 | 279.2 | 0.76 ± 0.19 | −1.68 ± 0.56 | 0.20 |

[a]for chemical structure of modified chitosans see Table 1; [b]concentration causing 50% fungal growth inhibition; [c]slope of log-concentration inhibition regression line; [d]intercept of regression line

mono-substitution. Moreover, the substitution of both the chlorine and fluorine atom dramatically increased the antifungal activity against *F. oxysporum* (compound 8).

The antifungal activity of the compounds against *P. debaryanum* (Table 2) illustrated that chitosan is still the least active compound. *N*-(*o*-chloro, *o*-fluorobenzyl)chitosan exhibited the most potent

Table 3. Effect of chitosan and its derivatives on spore germination of *A. alternata* and *F. oxysporum*

| Compound | R[a] | Concentrace (mg/l) | Spore germination (%) ± SE | Inhibition of spore germination (%) ± SE |
|---|---|---|---|---|
| *A. alternata* | | | | |
| Control | – | 0 | $98.61^{a}$ ± 1.39 | $1.39^{n}$ ± 1.39 |
| | | 125 | $86.00^{bc}$ ± 2.65 | $14.00^{lm}$ ± 2.65 |
| Chitosan | – | 250 | $76.50^{ef}$ ± 1.55 | $23.50^{ij}$ ± 1.55 |
| | | 500 | $49.70^{i}$ ± 2.61 | $50.30^{f}$ ± 2.61 |
| | | 125 | $67.34^{g}$ ± 3.27 | $32.67^{g}$ ± 3.27 |
| 1 | *o*-Cl | 250 | $50.71^{i}$ ± 3.01 | $49.30^{f}$ ± 3.01 |
| | | 500 | $20.32^{l}$ ± 3.85 | $79.68^{c}$ ± 3.85 |
| | | 125 | $79.04^{de}$ ± 1.47 | $20.96^{jk}$ ± 1.47 |
| 2 | *p*-Cl | 250 | $62.48^{gh}$ ± 0.95 | $37.52^{gh}$ ± 0.95 |
| | | 500 | $0.00^{n}$ ± 0.00 | $100.0^{a}$ ± 00.00 |
| | | 125 | $100.00^{a}$ ± 0.00 | $0.00^{n}$ ± 0.00 |
| 3 | *o*-F | 250 | $35.61^{j}$ ± 1.72 | $64.40^{e}$ ± 1.72 |
| | | 500 | $0.00^{n}$ ± 0.00 | $100.00^{a}$ ± 0.00 |
| | | 125 | $80.60^{de}$ ± 1.68 | $19.40^{jk}$ ± 1.68 |
| 4 | *p*-F | 250 | $59.70^{h}$ ± 0.87 | $40.30^{g}$ ± 0.87 |
| | | 500 | $0.00^{n}$ ± 0.00 | $100.00^{a}$ ± 0.00 |
| | | 125 | $84.53^{cd}$ ± 0.49 | $15.48^{kl}$ ± 0.49 |
| 5 | *o,p*-di Cl | 250 | $79.46^{de}$ ± 0.71 | $20.55^{jk}$ ± 0.71 |
| | | 500 | $0.00^{n}$ ± 0.00 | $100.00^{a}$ ± 0.00 |
| | | 125 | $88.13^{bc}$ ± 1.20 | $11.88^{lm}$ ± 1.20 |
| 6 | *o,o*-di Cl | 250 | $65.62^{g}$ ± 1.72 | $34.39^{h}$ ± 1.72 |
| | | 500 | $0.00^{n}$ ± 0.00 | $100.00^{a}$ ± 0.00 |
| | | 125 | $72.90^{f}$ ± 0.86 | $27.10^{i}$ ± 0.86 |
| 7 | *o,o*-di F | 250 | $12.48^{m}$ ± 1.55 | $87.52^{b}$ ± 1.55 |
| | | 500 | $0.00^{n}$ ± 0.00 | $100.0^{a}$ ± 0.00 |
| | | 125 | $90.90^{b}$ ± 1.90 | $9.10^{m}$ ± 1.90 |
| 8 | *o*-Cl, *o*-F | 250 | $27.08^{k}$ ± 1.47 | $72.92^{d}$ ± 1.47 |
| | | 500 | $0.00^{n}$ ± 0.00 | $100.00^{a}$ ± 0.00 |
| *F. oxysporum* | | | | |
| Control | – | 0 | $69.00^{a}$ ± 1.66 | $31.00^{i}$ ± 1.66 |
| | | 125 | $65.75^{a}$ ± 2.56 | $34.25^{i}$ ± 2.56 |
| Chitosan | – | 250 | $54.25^{bcd}$ ± 3.57 | $45.75^{fgh}$ ± 3.57 |
| | | 500 | $46.20^{ef}$ ± 2.24 | $53.80^{de}$ ± 2.24 |
| | | 125 | $42.50^{f}$ ± .23 | $57.50^{d}$ ± 3.23 |
| 1 | *o*-Cl | 250 | $12.89^{i}$ ± 1.49 | $87.11^{a}$ ± 1.49 |
| | | 500 | $8.15^{i}$ ± 0.29 | $91.86^{a}$ ± 0.29 |

Table 3 to be continued

| | | | | |
|---|---|---|---|---|
| | | 125 | $46.68^{def} \pm 1.13$ | $53.32^{def} \pm 1.13$ |
| 2 | *p*-Cl | 250 | $13.34^{i} \pm 1.17$ | $86.66^{a} \pm 1.17$ |
| | | 500 | $8.74^{i} \pm 1.54$ | $91.26^{a} \pm 1.54$ |
| | | 125 | $56.02^{bc} \pm 0.92$ | $43.98^{gh} \pm 0.92$ |
| 3 | *o*-F | 250 | $23.85^{gh} \pm 2.48$ | $76.16^{bc} \pm 2.48$ |
| | | 500 | $12.08^{i} \pm 1.48$ | $87.92^{a} \pm 1.48$ |
| | | 125 | $51.23^{bcde} \pm 2.19$ | $48.77^{efgh} \pm 2.19$ |
| 4 | *p*-F | 250 | $22.87^{gh} \pm 2.74$ | $77.14^{bc} \pm 2.74$ |
| | | 500 | $8.24^{i} \pm 1.03$ | $91.77^{a} \pm 1.03$ |
| | | 125 | $44.2^{ef} \pm 1.90$ | $55.79^{de} \pm 1.90$ |
| 5 | *o,p*-di Cl | 250 | $27.68^{g} \pm 1.90$ | $72.32^{c} \pm 1.90$ |
| | | 500 | $10.07^{i} \pm 1.97$ | $89.93^{a} \pm 1.97$ |
| | | 125 | $57.13^{b} \pm 0.93$ | $42.88^{h} \pm 0.93$ |
| 6 | *o,o*-di Cl | 250 | $25.60^{gh} \pm 1.04$ | $74.40^{bc} \pm 1.04$ |
| | | 500 | $9.98^{i} \pm 0.60$ | $90.02^{a} \pm 0.60$ |
| | | 125 | $48.53^{cdef} \pm 0.94$ | $51.48^{defg} \pm 0.94$ |
| 7 | *o,o*-di F | 250 | $29.00^{g} \pm 2.95$ | $71.00^{c} \pm 2.95$ |
| | | 500 | $11.13^{i} \pm 0.55$ | $88.88^{a} \pm 0.55$ |
| | | 125 | $48.45^{cdef} \pm 2.16$ | $51.55^{defg} \pm 2.16$ |
| 8 | *o*-Cl, *o*-F | 250 | $20.00^{h} \pm 2.97$ | $80.00^{b} \pm 2.97$ |
| | | 500 | $7.89^{i} \pm 1.08$ | $92.11^{a} \pm 1.08$ |

[a]for chemical structure of modified chitosans see Table 1; data are averages ± SE of four replications; values within a column bearing the same superscript are not significantly different ($P \leq 0.05$) according to the Student-Newman-Keuls (SNK) test

fungicidal activity ($EC_{50}$ = 155.7 mg/l), whereas *N*-(*o,o*-di fluorobenzyl)chitosan and *N*-(*p*-chlorobenzyl)chitosan were significantly the least active compounds among these derivatives ($EC_{50}$ = 1142 mg/l and 1174 mg/l, respectively). Moreover, the data showed that the substitution of a fluorine atom on the phenyl ring was more active than the substitution of a chlorine atom except compound 7. In addition, when we substituted one of the fluorine atoms in *N*-(*o,o*-difluorobenzyl)chitosan with a chlorine atom, the toxicity dramatically increased (see compound 7 versus 8).

When we regard the susceptibility of the microorganisms, another point deserves attention; the plant pathogenic fungi *F. oxysporum* and *P. debaryanum* are more susceptible to chitosans than *A. alternata*. The results obtained coincided that both the tested fungi *F. oxysporum* and *P. debaryanum* have no chitosan within their cell walls. This finding is in agreement with Allan and Hadwiger (1979), who suggested that the presence of chitosan within the cell wall of some fungi rendered those strains more resistant to the antifungal property of externally amended chitosan.

Recently, some of *N*-substituted chitosan derivatives were synthesised and their biological activities were evaluated against some plant pathogenic fungi including *Botrytis cinerea, Pyricularia grisea, F. oxysporum*, and *P. debaryanum* (Rabea *et al.* 2005, 2009; Badawy 2008, 2010). *N*-(*o,o*-dichlorobenzyl)-chitosan within a series of *N*-(benzyl)chitosan derivatives was the most active compound against *B. cinerea* with $EC_{50}$ of 520 mg/l (Rabea *et al.* 2005). In addition, *N*-(*o,p*-diethoxybenzyl)chitosan

was the most active one with $EC_{50}$ of 400 mg/l and 468 mg/l for *F. oxysporum* and *P. debaryanum,* respectively (Rabea *et al.* 2009).

Among five *N*-(heterocyclic)chitosan derivatives (Badawy 2008), *N*-((5-methylfuran-2-yl) methyl)-chitosan was the most active against *P. grisea* ($EC_{50}$ = 919 mg/l), while *N*-(benzo[*d*][1,3]dioxol-5-yl-methyl)chitosan and *N*-(methyl-4*H*-chromen-4-one)chitosan exhibited the most potent fungicidal activity against *P. debaryanum* ($EC_{50}$ = 438 mg/l and 424 mg/l, respectively) and *F. oxysporum* ($EC_{50}$ = 1549 mg/l and 1106 mg/l, respectively).

In addition, it has been generally documented that the biological activity of chitosan and its derivatives depends on the degree of deacetylation, chemical modification, degree of substitution, position of a substituent on the glucosamine units and the target organism (Jeon *et al.* 2001; Gerasimenko *et al.* 2004). Our results are in agreement with this statement, when the chemical modification of chitosan molecule led to a high increase in fungicidal activity against the tested fungi compared to unmodified chitosan. Moreover, the position of substitution (*ortho-* and/or *para*-positions) was very effective in antifungal activity. These variations suggested to lead to two different mechanisms of chitosan molecule and target microorganism interaction: the first is the adsorption of chitosans to cell walls leading to the cell wall covering, membrane disruption and cell leakage; the second is the penetration of chitosans into living cells leading to the inhibition of various enzymes and interference with the synthesis of mRNA and proteins (Chircov 2002; Rabea *et al.* 2003; Zheng *et al.* 2003).

In the spores of *A. alternata* (Table 3), the results showed the complete inhibition (100%) of fungal spores at the high concentration tested (500 mg/l) among all compounds except compound 1 and chitosan. It can also be seen that the compound of *N*-(*o*,*o*-difluorobenzyl)chitosan (7) was significantly the most potent one (27.10%, 87.52%, and 100% inhibition at 125 mg/l, 250 mg/l and 500 mg/l, respectively). In regard to the effect of substituent and position on the phenyl moiety, it can also be noticed that the fluorine atom enhanced the antispore germination activity more markedly than the substitution of a chlorine atom (compounds 1, 2, 5, and 6 versus 3, 4, and 7).

Data on the spore germination of *F. oxysporum* (Table 3) demonstrated that the spores were affected significantly by all compounds compared to the control. In general, compared with the activity of chitosan, all derivatives were high in the inhibition of spore germination. The results revealed that a high inhibition (> 87%) of fungal spores was observed at the high concentration tested (500 m/l) among all compounds and there is no significant difference among the compounds at this concentration. *N*-(*o*-chlorobenzyl)chitosan (1) and *N*-(*p*-chlorobenzyl)chitosan (2) were significantly the most potent compounds (57.50%, 87.11% and 91.86% and 53.32%, 86.66% and 91.26% inhibition at 125 mg/l, 250 mg/l and 500 mg/l, respectively). Based of the statistical analysis, the compounds can be divided into four groups as follows: compounds 1 and 2 > 8 > 3, 4 and 6 > 5 and 7.

The results obtained are in agreement with those published recently (Rabea *et al.* 2009). They reported that a different group of *N*-(benzyl)chitosan derivatives exhibited high inhibition percentages (> 90%) on the spore germination for *F. oxysporum* at 1000 mg/l. Hernández-Lauzardo *et al.* (2008) added that the spore germination of *R. stolonifer* was affected by chitosan of different molecular weights ($1.74 \times 10^4$, $2.38 \times 10^4$ and $3.07 \times 10^4$ g/mol). They found that chitosan of $1.74 \times 10^4$ g/mol and $2.38 \times 10^4$ g/mol markedly reduced the spore germination, but no significant effects were found among the tested concentrations (1.0 g/mol, 1.5 g/mol and 2.0 mg/l). However, they observed the complete inhibition of spore germination with a chitosan of $3.07 \times 10^4$ g/mol. Badawy (2010) also reported that among the synthesised chitosan derivatives, *N,N,N*-(dimethylpentyl)chitosan and *N,N,N*-(dimethylhexyl)chitosan markedly reduced the spore germination of *B. cinerea* with significant effects (96.54% and 97.08% inhibitions, respectively at 250 mg/l).

Consequently, it is essential to carry out extra basic studies on the mode of action and the effect of the physicochemical property of chitosan derivatives on various stages of development of fungi.

## CONCLUSIONS

The results reported here demonstrate the biological activity of chemically modified chitosans against the most important economic plant pathogens. Chitosan derivatives markedly inhibited the growth of the bacteria *A. tumefaciens* and *E. carotovora* with MIC values ranging between 480 and 1700 mg/l and their activity was higher than that of the unmodi-

fied chitosan. Most of these compounds showed promising fungicidal activity against the tested fungi and *N*-(*p*-fluorobenzyl)chitosan was the most active against *A. alternata,* while *N*-(*o*-chloro,*o*-fluorobenzyl)chitosan exhibited the most potent fungicidal activity against *F. oxysporum* and *P. debaryanum*. In addition, all the chitosan compounds had good antimicrobial activity at a high concentration (500 mg/l) against fungal spores. This result demonstrated that the chemical modification of chitosan molecule enhanced the biological activity against some plant pathogenic bacteria and fungi. Therefore, this class of compounds can be used to control a number of plant pathogens that cause destruction of crops and vegetables. In future investigations, further studies as in situ experiments are needed to investigate the effects of these compounds on bacteria and fungi.

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